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Original Article

TOPICAL DRUG DELIVERY OF GOSSYPIN FROM PRONIOSOMAL GEL FORMULATIONS: *IN VITRO* EFFICACY AGAINST HUMAN MELANOMA CELLS

JAMPALA RAJKUMAR[1*], G. V. RADHA[1], S. GANAPATY[1]

[1]GITAM Institute of Pharmacy, GITAM (Deemed to be University), Rushikonda, Visakhapatnam 530045, Andhra Pradesh State, India
Email: rajpharma_jampala@yahoo.com

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ABSTRACT

Objective: This work aimed to establish and formulate the gossypin proniosomal gel drug delivery system.

Methods: Gossypin-loaded proniosomal gels (GPG) was prepared using specific non-ionic surfactants (Spans), followed by particle size (PS), entrapment efficiency (percent EE), *in vitro*, ex-vivo drug release, and *in vitro* efficacy of Gossypin against human melanoma cells (A-375).

Results: The results showed that the percentage EE for the GPG is appropriate (81.3 %–95.5 %) and they are Nano-sized (189.3–912.0 nm) and the gels diffusion provided the desired sustaining effect for GPG-F7 formulation (75.5 percent). The GPG reported cell viability of 14.9±2.3 percent compared with 16.1±1.1 percent for free Gossypin at the maximum dose of 100 µg/ml for A-375 human melanoma cells after 24 hr incubation time. No major changes were seen in the percentage EE, PS of GPG after storage for 90 d, in the physical stability report.

Conclusion: The results obtained suggest that the proniosomal drug delivery system can enhance the flux to the skin and achieve the ideal Gossypin sustainability effect. Consequently, the use of proniosomal gel may be advantageous with regard to the topical delivery of Gossypin for melanoma treatment management.

Keywords: Proniosomal gel, Gossypin, A-375 human melanoma cells, Cytotoxicity, Topical delivery, Skin cancer

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INTRODUCTION

The most dangerous diseases among different cancers of the skin are melanoma. Cutaneous melanoma accounts for about 3 percent of all skin cancers and 75 percent of malignant cancer causes higher mortality rates [1]. Chemotherapy, cell therapy, immunotherapy and hormone therapy are different cancer treatments, with cancer varying from 5-10 % annually [2]. Actinic and solar keratosis was primarily seen in skin conditions, and numerous authors reported their treatment [3]. Aside from current therapies for skin cancer, there is an urgent need for new drug molecules. Although new therapies (ipilumab, pembrolizumab and nivolumab) display improved chronic responses, the key disadvantage from such therapy effectiveness is the severe adverse effects [4, 5]. Phytochemicals are supplied in the treatment of low-cost and low-toxicity skin cancer as a dietary supplement. The diagnosis of skin cancer now plays a prominent role in the delivery of topical drugs [6]. The polyphenolic compound in herbal foods is flavonoids. There are 400 different types of organic behaviors, such as anti-tumor and anti-inflammatory acts [7].

Gossypin belongs to the glucosyl flavone class found in the malvaceae family's flora of hibiscus vitofolius [8]. This is water-soluble due to the existence of glucose moiety [9]. Gossypin (10 and 100 mg/kg) has been reported for use with apoptosis in the treatment of human melanoma (A-375, xenografts) with BRAFV600E, which is a mutation of BRAF gene [10] formerly known as serine/threonine-protein kinase. It is found to be a new anti-carcinogenic agent for the treatment of BRAFV600E kinase and CDK4 (cycline-dependent kinases) inhibitory melanoma [11]. In addition to antioxidant, analgesic and hepatoprotective activities, it has a defensive role against beta-amyloid-induced toxicity [12]. The growth factor of beta-activated kinase-1-mediated NF (nuclear factor) kappaB pathways, penta hydroxylic glucocyle flavone, inhibits the apoptosis and subsequently suppresses osteoclastogenesis [13, 10]. Gossypin has been confirmed to have anti-cancerous papillomas sustained in the rodent model DMBA (7, 12-dimethylbenz (*a*) anthracene) [14, 15]. The perfect medicine for skin conditions reaches deeper skin layers. The technique involves assessing the concentration of medicines in the epidermis and dermis in order to ensure an successful distribution [16].

Localization of drugs improve long-term effectiveness [1]. This medicine is encapsulated in a vesicular medication supply to enhance the transdermal supply of liposomes, ethosomes, and transferosomes [17], but this medicinal substance may become unstable because of accumulation, hydrolysis, fusion and sedimentation during storage [18]. The delivery mechanisms of proniosomes solved vesicular system stability problems. Proniosomes structures consisting of non-ionic surfactants forming niosomes through water absorption, producing an occlusive state, and serving as a potential route of transdermal delivery are considered to be semi-strong gel-like composites [19]. The main goal was to explore new approaches using non-ionic surfactants using different HLB (Hydrophilic-Lipophilic balance) values. The intrinsic properties of tensile substances determined by phase transition temperature, surfactant form etc. In vitro drugs, particulate scale, ex-vivo skin penetration, in vitro cytotoxicity and stability were also studied.

MATERIALS AND METHODS

Materials

Gossypin was supplied as a gift sample from Prof. Ganapaty, Principal GIP-GITAM (Deemed to be University, Visakhapatnam, A. P (India). Cholesterol procured from Finar chemical laboratory, soya lecithin obtained from sigma limited chemicals (Hyderabad, India). Span20, Span40, Span60, and Span80 were collected from Molychem laboratories, Thane (India). Dialysis membrane (12,000-14,000 M. W cut off) was purchased from Himedia Laboratories Pvt. Ltd., Mumbai, (India). All other solvents and chemicals were of scientific-analytical grade. A-375 cells of human melanoma were collected from the National centre for cell science, Pune (India).

Animals

Swiss albino mice (25±30 gr) 6-8 w old were taken from the disease-free animal house of the National Institute of Nutrition, Hyderabad (India). Animals were kept in groups of four in cages at room temperatures between 20 °c and 25 °c and had free access to food and water. All research and testing activities have been reviewed and approved by the Institutional Animal Ethics Committee (IAEC/GIP-1287/GVR-F/2019).

Methods

Preparation of gossypin proniosomal gel (GPG)

The proniosomal gel has been prepared with different grades of non-ionic surfactant, lecithin, and cholesterol using a coacervation separation process [20, 21]. The batch amount of gossypin was precisely weighed together with surfactant; lecithin and cholesterol were mixed with 2.5 ml of ethanol in a wide mouth glass tube. The contents are heated in a water bath at 65±3 °C for 5 min. In order to achieve transparent dispersion, 1.6 ml pH-7.4 phosphate buffer solution was added to the above mix under constant heating conditions. The transparent dispersion was cooled until a gel-like structure (proniosomal gel) had been formed. During the preparation, various grades of spans were used to test their effect on formulation characteristics. Different preparations were analysed with varying amounts of surfactant and lecithin. Cholesterol is an important factor which gives stability and penetrability to the vesicles. The compositions produced are listed in (table 1).

Characterisation of gossypin proniosomal gel (GPG)

Particle size distribution and zeta potential

The equipment for the Malvern-zeta sizer (Nano ZS90) was used to calculate the average size, size and zeta potential by the dynamic light distribution method. The prepared proniosomal gel was dispersed up to 0.2 g in 10 ml of the 7.4 pH phosphate buffer solution. The prepared solution was tested at a refractive index of 1,333 for the size and poly dispersibility index (PI) of 25 °C. The PI was developed as a measure of homogeneity [22]. PI received as:

$$\mathrm{PI} = (\text{Standard Deviation/Vesicle size})$$

Small PI values (<0.1) indicate a standardized population, while PI values>0.3 are too high.

Encapsulation efficiency (% EE)

The prepared proniosomal gel (0.2 gr) has been dispersed with 10 ml of pH 7.4 phosphate buffer solution for trapping efficiency. Sonication in a bath sonicator for 30 min was subjected to a watery dispersion of niosomes [19, 40]. The gossypin containing niosomes has been centrifuged for 45 min to separate gossypin at 9000 rpm at 4 °C. The supernatant was collected and the concentration of gossypin was analysed by UV spectrophotometer at 278 nm from the following equation: The proportion of drugs encapsulated was determined:

$$\%\,\mathrm{E.E} = 1 - \frac{(\text{Unentrapped drug})}{(\text{Total drug})} \times 100$$

Differential scanning calorimetry (DSC)

The possible interactions between gossypin and vesicular ingredients, based on the thermal properties of a pure drug, were evaluated using diamond DSC (Mettler star sw8.10) by Differential Scanning Colorimetry (DSC) [23]. A maximum of 50 °C min-1 to 400 °C min^{-1} was conducted under nitrogen flow of 25 ml min^{-1}. GPG-7 was selected with the highest percentage EE, and for a DSC evaluation, samples of 4 mg per niosome from gossypin, span 60, empty and drug-loaded proniosomes were sent. The precisely weighed sample sum was crimped in the DSC aluminium pot and samples were analysed at a heating rate of 10 °C/min at a temperature of 400 °C.

Fourier transform infrared (FTIR) spectroscopy

Gossiping powder was pressed into a pell*et al.* ong with KBr (potassium bromide) using a hydraulic press. GPG-7 final optimized formulation IR spectrum of drug gossypin was tested on Fourier transform infrared spectroscopy (FTIR) in the 4000-400 cm^{-1} wave range [23].

Transmission electron microscope

The morphological examination of the prepared niosomal gel was analysed with TEM. Hydrating the proniosome gel (0.2 gr) with 10 ml phosphate buffer solution (pH 7.4) was used to prepare the sample. A scatter droplet was placed on a carbon-coated copper grid and left to stay for around 1 minute on the carbon substrate. The dispersion was also extracted with a little Whatman paper. A 1% phosphorous tungsten acid solution was applied and again, an excess solution was separated by a tip of whatman paper. Samples were allowed to dry for 10 min at room temperature for inspection after being labelled [14].

***In vitro* drug release study**

Gossypin release from Proniosomal gel was determined by the use of a technique of dialysis membrane diffusion [24, 25]. An open glass tube with a diameter of 4,153 cm^2 and an effective length of 8 cm has been closed one end with the dialysis membrane previously soaked overnight in the pH phosphate buffer 7.4, which acts as a rate-limiting membrane. The glass tube toward the donor compartment was filled with 5 mg gel formulation. During a beaker that contains 40 ml of pH 7.4 phosphate buffer, the glass tube was positioned which acts as a receptor compartment medium. The entire assembly was set in such a way that the lower end of the membrane-containing channel, which had just met the surface of the diffusion medium after having gel (1-2 mm deep). The receptor department temperature was held at 37±2 °C, and thus the medium was agitated at 100 rpm speed using a 24 h magnetic stirrer. Samples were obtained at different periods of time (1, 2, 3, 4, 5, 6, 7, 8, 12, 18 and 24 h). Aliquots of 5 ml samples were regularly removed and replaced by an equal amount to maintain sink conditions. Using a validated analytical approach (n=3), the samples obtained were analysed.

***Ex-vivo* skin permeation and drug retention study**

The research analysis was carried out after approval by the ethical committee of the institutional animals (approval no: IAEC/GIP-1287/GVR-F/2019). Swiss albino mice (25±30 gr) were used in the study, the animals were housed in separate cages and held under controlled temperature conditions and the rats had free access to water and food before they were sacrificed in compliance with the guidelines for skin harvesting Euthanasia and carcass [14]. Using extra amount of anaesthetic ether, the rats were sacrificed. Once the skin was surgically extracted, hair on the dorsal side was cut with a hair clipper taking strict precautions not to damage the skin. A heat separation technique was used to prepare the epidermis [22, 26]. It involved soaking up the whole abdominal skin in the water at 60 °C for 45 seconds, followed by thorough removal of the epidermis. Dried water, wrapped in aluminium foil and held until further use at-20 °C [27] (used within 2 w of preparation). Ex-Vivo permeation experiments were performed using unjacketed Franz vertical diffusion cells with a 4,153 cm^2 diffusional surface area and 20 ml of the receptor cell length. The skin was brought to room temperature and placed between the Franz diffusion cell's donor-receiver compartments, where the stratum corneum side faced the donor compartment [21]. The skin was allowed to stabilize for 1 hr before dosing, and a formulation equivalent to 5 mg of gossypin was injected on the skin's dorsal side. The receptor compartment containing pH-7.4 phosphate buffer (containing 0.02 percent w/v of ethanol to retard microbial growth) was kept under continuous stirring up to 24 h at 32 °C on the skin surface [28]. At 300 rpm, the receiver compartment was continuously stirred [29]. The donor chamber and the sampling port were covered with a lid to avoid evaporation during the test. Aliquots of 5 ml were regularly removed at different time intervals (1, 2, 3, 4, 5, 6, 7, 8, 12, 18 and 24 h) and replaced by equivalent volume to preserve the receptor's constant phase volume. Same test with pure drug was performed. The samples were diluted sufficiently and the amount of the drug was measured at 278 nm (n=3) using the UV-Visible spectrophotometer. The substance stored in the skin was calculated with a minor change following the procedure [30]. The experiment was performed after the permeation tests were done (24 h) [31]. The gel present on the compartment cell was removed, washed with PBS and methanol (1:1 v/v) and the UV absorbance was set at 278 nm. The residual skin was sliced into tiny bits to determine the amount of drug in the viable skin (epidermis and dermis). They were poured into a conical flask containing 50 ml of PBS (7.4 pH) and methanol (1:1 v/v), put on for 48 h to the rotary shaker. The solution was then filtered using UV Visible Spectrophotometer at 278 nm, and the amount of drug extracted from the skin sample was determined.

Skin permeation data analysis

The cumulative amount of material that was permeated into a unit region of the skin was plotted as a function of time [32-34].

Flux

The Steady-State Flux was determined using slope of the graph.

$$Jss = (dQ/dt) * (1/A)$$

Where Jss = Steady State Flux (µg/cm^2/hr), A=Surface area, dQ/dt = Cumulative quantity permeated per unit area per unit time.

Permeability coefficient (Kp)

The following equation was used to calculate the coefficient of permeability, which describes the relation between the flux and the initial drug charge [35].

$$K_p = J_{ss}/C_{donar}$$

Where, Kp =Permeability co efficient (cm/hr), Jss = transdermal flux, C= Initial concentration of drug donor compartment [36].

***In vitro* cytotoxicity study of gossypin proniosomal gel (GPG)**

In vitro cytotoxicity for free drugs and drug-charged gels was estimated by 3-(4, 5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) assay. Briefly, A-375 human melanoma cells were seeded into 96-well plates in 100 µL Dulbecco Modified Eagle Medium at a density of 1 X 104 cells per well and incubated overnight. Cells were then exposed to a sequence of free drugs and drug-loaded gels at a change in the strength of 0-100 µg/ml for 24 h, correspondingly using A-375 human melanoma cells as a trigger untreated. After the incubation period, the media was removed and the cells were treated with an MTT solution (50 µL; 5 mg/ml) in the Dulbecco Modified Eagle Medium serum-free for 4 h. The solution has been removed and 150 µL of dimethyl sulfoxide (DMSO) has been added to each well. The plate was previously incubated at room temperature for 30 min. Absorption intensity was measured using a 590 nm UV-Vis spectrophotometer with a reference wavelength of 620 nm. The following equation expressed the viability of cells [34].

$$\text{Cell viability (\%)} = \text{Absorbance of}\frac{\text{sample}}{\text{Absorbance}}\text{of control} \times 100$$

Stability studies

The preparations were packed in glass vials undergoing stability tests at accelerated storage conditions (40 °C±2 °C), room temperature (25±2 °C) and in a refrigerator (2-8 °C) for three months. Samples were evaluated for entrapment efficiency and size at definite intervals (1,2 and 3 mo). Formulations that retain entrapping capacity for 3 mo are known as long-lasting formulations. Samples were taken and hydrated with phosphate-buffered saline solution (pH 7.4) and analyzed under an optical microscope for any evidence of product crystallization. Additionally, the samples were percentage-tested for particle size and gossypin retention [35, 36, 41].

RESULTS AND DISCUSSION

Preparation of gossypin proniosomal gel (5-FUPG)

In the present investigation, proniosomal gel-loaded gossypin was prepared using non-ionic surfactants using coacervation techniques. Prepared niosomes were evaluated for particle size, shape, entrapment efficacy, *in vitro*, ex-vivo drug release and efficacy against human melanoma cells. (table 1) Shown Composition of 1% gossypin proniosomal gel (GPG) formulations.

Table 1: Composition of 1% gossypin proniosomal gel (GPG) formulations

Formulation	Gossypin (mg)	Span20 (mg)	Span40 (mg)	Span60 (mg)	Span80 (mg)	Lecithin (mg)	Cholesterol (mg)
GPG-1	10	234.5	-	-	-	703.5	52
GPG-2	10	-	234.5	-	-	703.5	52
GPG-3	10	-	-	234.5	-	703.5	52
GPG-4	10	-	-	-	234.5	703.5	52
GPG-5	10	469	-	-	-	469	52
GPG-6	10	-	469	-	-	469	52
GPG-7	10	-	-	469	-	469	52
GPG-8	10	-	-	-	469	469	52
GPG-9	10	703.5	-	-	-	234.5	52
GPG-10	10	-	703.5	-	-	234.5	52
GPG-11	10	-	-	703.5	-	234.5	52
GPG-12	10	-	-	-	703.5	234.5	52

Particle size distribution and zeta potential

The results of newly reconstituted dispersions of gossypin proniosomal developed formulation are shown in table 2. All formulations were found in the nanoscale range between 189.3 nm (GPG-7) and 921.9 nm (GPG-9) with a low polydispersity index ranging from 0.108 (GPG-6) to 0.199 (GPG-9), indicating homogenous dispersion. In addition, the measured zeta potential values of the formulated proniosomes were negatively charged, with zeta potential loads ranging from-22.65 mV to-55.52 mV showing good physical stability of the niosomal dispersion.

Spans are well-known non-ionic surfactants used as vesicles for the treatment of niosomes and proniosomes. When assessing the different grades of Spans, e. g. 20, 40, 60 or 80, it was found that their nature significantly affected the final vesicle sizes [9].

Table 2: Particle size, polydispespersity index and zeta potential analyses of gossypin proniosomal gels

Formulation	Particle size(nm)	Polydispersity index	Zetapotential (mV)
GPG-1	912.0	0.124	-29.54
GPG-2	657.3	0.186	-35.86
GPG-3	224.6	0.136	-47.45
GPG-4	554.6	0.137	-22.65
GPG-5	456.2	0.121	-49.82
GPG-6	758.6	0.108	-31.21
GPG-7	189.3	0.186	-55.52
GPG-8	652.4	0.186	-36.21
GPG-9	921.9	0.199	-27.93
GPG-10	463.8	0.154	-32.42
GPG-11	235.4	0.178	-50.81
GPG-12	545.1	0.185	-25.80

Data expressed mean±SD (n= *3*)

Encapsulation efficiency (% EE)

In the design of vesicular formulations, encapsulation efficiency is one of the essential parameters. The effectiveness of vesicle encapsulation depends on the type and amount of surfactant used in bilayer formation and on the amount of cholesterol and lecithin [37]. Efficiency of encapsulation tests of formulations are shown in table 4. For formulations prepared with Span 60, higher encapsulation efficacy than with Span 20, Span 40 and Span 80 were observed. Span 60's longest saturated alkyl chain shows a higher phase transition temperature (Tc), which represents the percentage of encapsulation efficiency. For Span 60 formulations of Span 20, 40 and 80, the vesicle size was also less. The literature also indicates that, as hydrophobicity of the monomer of surfactants increases, it decreases the size of the vesicle. Both span forms are of the same head group, but alkyl chains are distinct. The length of the alkyl chain increases the encapsulation efficiency. The order of encapsulation efficiency was observed for different grades of Span 60 (C18)>Spain 40(C16) Spain 60 and 80 have identical head classes, but Spain 80 has an alkyl chain which is unsaturated. Span 80 is the lowest in the comparison spans (16 °C for span20, 42 °C for span 40, and 53 °C for span 60). Spain 80 is of the highest transition temperature, Tc=12 °C). The highest transition temperature in the process in Span causes the medicine to be entrapped the lowest and vice versa. In the range of 81.3% to 94.4% and in the intermediate stage of the surfactant concentration EE, specifically, was found to be within the range of 81.4% to 95.5%. Whereas the surfactant EE level was found to be between 81.3% and 91.8% at a higher level. The lower, mid and higher levels of lecithin content were shown to increase gradually in EE, which is usually added to improve the stability of the formulation.

Differential scanning calorimetry (DSC)

Fig. 1(a) and 1(b) below display gossypin and drug-loaded niosomes with DSC thermograms. Gossypin exhibited an endothermic peak corresponding to their melting temperatures at 228.5 °C, respectively. Proniosomal DSC thermogram showed a high endothermal peak at 129 °C, suggesting the association of span 60, cholesterol, and lecithin molecules. This supports double-layer structure of the vesicle. No gossypin peak in the drug-loaded formulation has been identified, suggesting the amorphous nature of the drug. This observed finding may be due to the complete solubility of the drug in the vesicles and may account for the increased entanglement in formulations of gossypin.

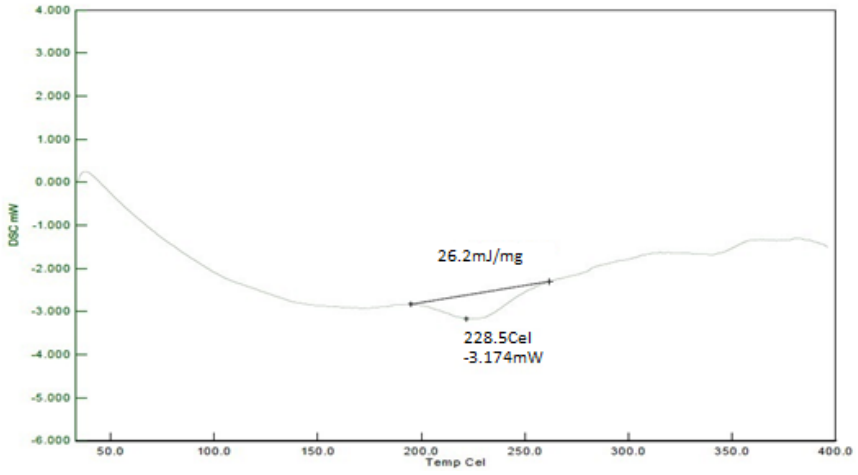

Fig. 1: a) DSC thermograms of gossypin

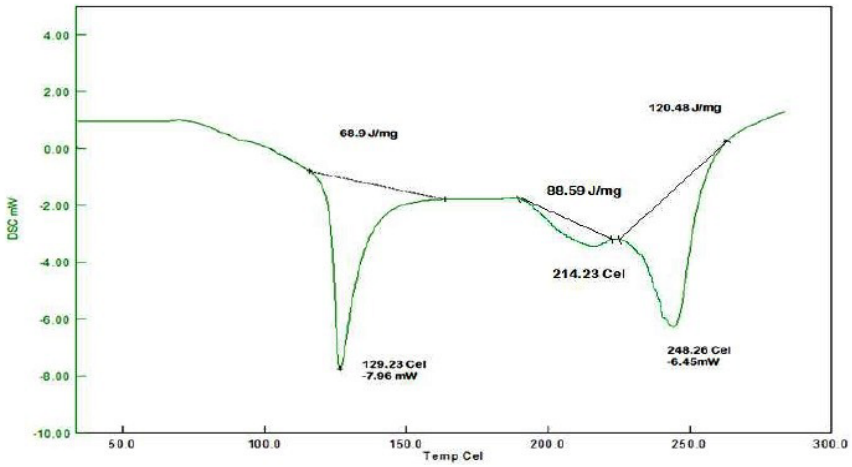

Fig. 1: (b) DSC thermograms of GPG-7

Fourier transforms infrared (FTIR) spectroscopy

FTIR study of the selected formulation (GPG-7) prepared with the non-ionic surfactant, i.e. Span 60, soya lecithin, cholesterol and drug. The spectrum peak points of the formulation were similar to that of pure gossypin, which clearly indicates that there is no drug or excipient interaction. The FTIR spectra of pure gossypin and the formulation were given in the following section. FTIR images have been shown in (fig. 2 and table 4.) describes the FTIR spectrum of peak points of the pure drug and the prepared formulation (GPG-F7).

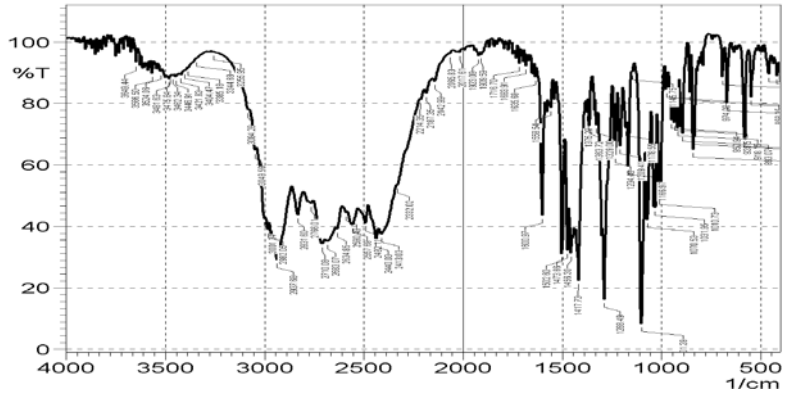

Fig. 2: (a). FTIR spectrum of gossypin

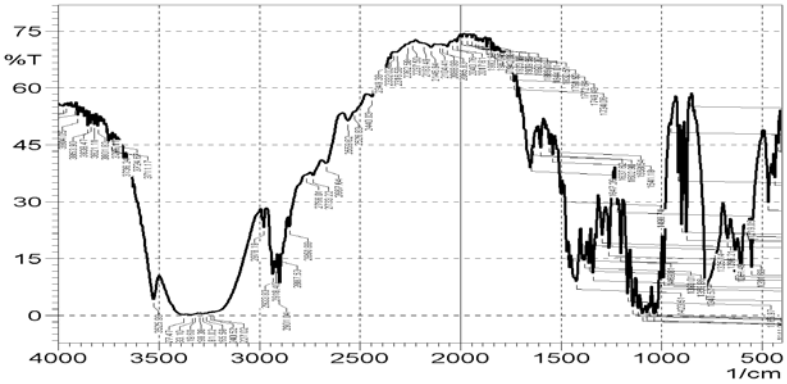

Fig. 4: (b). FTIR spectrum of GPG-7

Table 3: **Describes the FTIR spectrum peak points of pure Gossypin and the prepared formulation (GPG-7)**

Pure gossypin	**Gossypin (GPG-7)**
Wavenumber cm^{-1}	
3524(alc. O-H)	3525
1635(C=O)	1637
1600(C-C)	1602
2982(SP^3C-H)	2978
3381(phenolic O-H)	3385
1502(Sp^2C-H)	1498

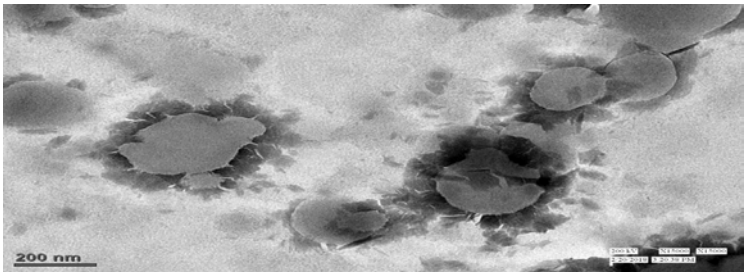

Fig. 3: TEM image of GPG-7

Transmission electron microscope

(fig. 3) Illustrates the presence and surface morphology of vesicles following hydration with distilled water for GPG-7. As a fiddle with a smooth surface, the inspected vesicles appeared well known, round fit.

***In vitro* drug release study**

The results of the *in vitro* release of gossypin from different formulations are shown in (table 4 and (fig. 4). The combination of gossypin in all proniosomal formulations showed a noteworthy slower release profile compared to the control of gossypin release in 12 h. The initial release was expected to be rapid due to gossypin disorption from the surface of the proniosomes. Controlled slow release was expected due to the diffusion of the swollen proniosomal bilayer. Proniosomes composed of Span60 indicated a higher release rate than those prepared for the use of non-ionic surfactants, Span40, Spam20 and Span80. Span20 and Span80 have lower phase transition temperatures and a less penetrable rigid

bilayer structure than Span60 and Span40, which frames a progressively porous liquid bilayer [9, 26]. Expanding the lecithin content from 234.5 to 703.5 mg resulted in an increasingly unblemished lipid bilayer as an obstruction to drug release. Increased concentration of lecithin decreased leakage and permeability of gossypin, delayed release of trapped drugs from the vesicles and resulted in a noteworthy slow release profile. In addition, the best release profile of the different formulations was observed using Span60 with a mean concentration of 469 mg. Formulation F7, containing Span60 as a surfactant, showed more *in vitro* release of drugs, i.e. 75.5 percent of all surfactants. The release rate was also dependent on noisy membrane fluidity as a function of either acyl chain length and saturation or cholesterol content. The optimized formula F7 based on Span60 was added to stability based on *in vitro* characterizations (table 6). When comparing the release outlines of the control formulation with that of the proniosomal formulation, a significant delay in drug release was seen in proniosomes. Long-term treatment with prolonged-release patterns was required to treat the targeted site drug delivery, in particular, the treatment of skin cancer of the type of topical disease [34]. Release studies were evaluated for release of the kinetic mechanism using Zero order, First order, Higuchi, Korsmeyer-Peppas models. Kinetic analysis of all formulation profiles followed a diffusion-controlled mechanism with an initial rapid-release phase followed by slower releases. Release of the GPG-7 batch, fitted to the Higuchi matrix equation, showing a high R2 value (0.98015). It can therefore, be determined that the release of gossypin from the proniosomal gel formulation was based on the diffusion-controlled mechanism of the Higuchi matrix. As a result, the release of the drug from this matrix may have been affected by the diffusion mechanism.

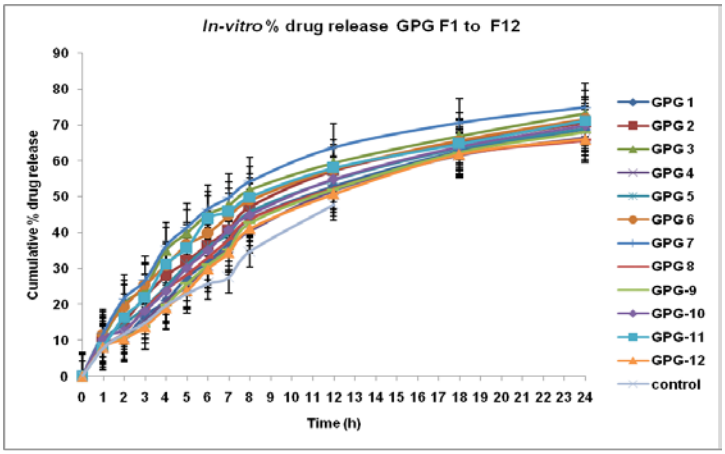

Fig. 4: *In vitro* drug release of proniosomal gels (GPG-1 to GPG-12) and control, data expressed mean±SD (n= 3)

Ex-vivo skin permeation and drug retention study

Proniosomes that permeates through the skin to form niosomal vesicles should be hydrated before the drug is released. Many mechanisms may explain the ability of niosomes to modulate drug transmission through the skin, including (i) adsorption and fusion of niosomes to the skin surface that will promote drug permeation, (ii) vesicles act as penetration enhancers to minimize the barrier properties of stratum corneum, and (iii) niosomal lipid bilayers act as a drug membrane barrier. Interestingly, no lag step could be detected and gossypin could be detected in the receptor medium within one hour, suggesting that all processes (water permeation from the receptor compartment to the skin, proniosomal conversion to niosomes, gossypin release from the reconstituted niosomes and its permeation) occurred quite quickly [38, 39].

Gossypin major permeation occurs only when the drug was released from the niosomes and formed with skin fluid after hydration of the proniosomal gel. The penetration data obtained from different proniosomal formulations based on surfactants compared with an equivalent concentration of the drug regulation. The findings showed that, in the first cycle, the amount of drug permeated through the skin was shown higher. Penetration rate was sluggish afterward for all formulations of the vesicles. Results shown in table 4 and fig. 5 seem to indicate that proniosomal formulations significantly delayed the penetration of the drug through the skin. Proniosomes were identified with a major increase in drug retention in the skin as they consisted of cholesterol, lecithin and a surfactant. The optimized formulation GPG-7 (span 60) showed a 65.5 percent higher drug release than the other formulations with Span 20, Span 40 and Span 80.

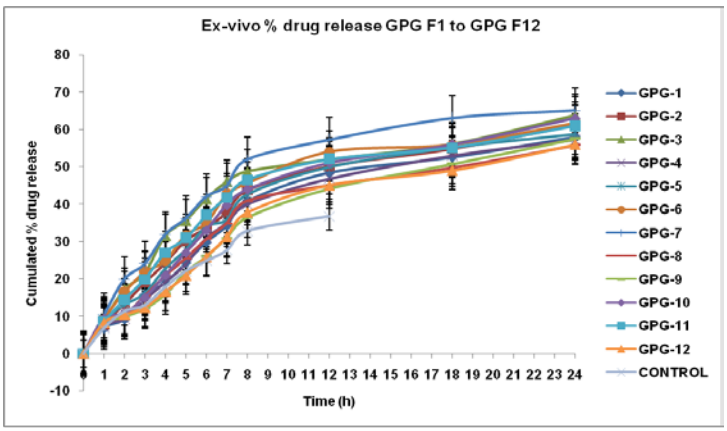

Fig. 5: Ex-vivo drug release of proniosomal gels (GPG-1 to GPG-12) and control, data expressed mean±SD (n= 3)

Table 4: Entrapment efficiency, *in vitro* and ex-vivo percentage cumulative drug release of gossypin proniosomal gels

Formulation	Entrapment efficiency (%)	*In vitro* percentage cumulative drug release (24 h) (%)	*Ex-vivo* percentage cumulative drug release (24 h) (%)
GPG-1	83.2	69.1	58.9
GPG-2	88.3	70.2	60.8
GPG-3	94.4	73.8	63.2
GPG-4	81.3	66.9	56.9
GPG-5	82.9	69.8	58.4
GPG-6	90.6	71.2	61.4
GPG-7	95.5	75.5	65.5
GPG-8	81.4	66.2	56.2
GPG-9	82.2	67.9	57.9
GPG-10	91.8	70.4	62.8
GPG-11	90.2	70.8	61.3
GPG-12	81.3	65.8	55.7

Data expressed in mean±SD (n=3)

Skin permeation data analysis

Even the parameters of proniosomal skin permeation, as well as drug suspension, were measured at a medication concentration equivalent. The flux value obtained from the GPG-7 proniosomal gel (20.3µg/cm²/h) was found to be 2.7 times higher than that of the suspension drug (7.6µg/cm²/h) (table 5). Gossypin's comparatively low suspensive skin permeability was attributed to its lower aqueous solubility, while the high proniosomal permeability resulted in improved penetration of the gossypin surface. Better transdermal flux without a lag step of the proniosomal gel may result from one or more wing mechanisms as previously mentioned, i.e. increased gossypin solubility, high association of drugs with vesicle bilayers, increased vesicle partitioning into the stratum corneum, and elasticity of the vesicle membranes [35].

Table 5: Permeation parameters of drug dispersion and proniosomal formulation

Formulation	Gossypin permeated through skin at 24 h (%)	Total gossypin accumulated to skin (%)	Flux (µg/cm²/h)
GPG-5	11.6	19.8	14.5
GPG-6	12.5	22.6	17.6
GPG-7	16.3	35.3	20.3
GPG-8	14.7	29.3	12.5
Drug dispersion	56.3	15.5	7.6

Data expressed in mean±SD (n=3)

In vitro cytotoxicity study of gossypin proniosomal gel (GPG)

The *in vitro* cytotoxicity of drug-carried gels has been tested in human melanoma cell lines A-375. Gels without active substances with the same concentrations of polymers as drug-induced gels were used as control. Cells were incubated in the presence of free-drug and drug-loaded gels for 24 h and were tested with the MTT assay. After incubation with various formulations, the viability of A-375 human melanoma cells was shown in fig. 6. Gossypin loaded gels (GPG) had cell practicality of 14.9±2.3% compared to 16.1±1.1%. The formulations are incubated 24 h long and display higher cytotoxicity in human melanoma cells A-375. We have tried medication-free gels to show that the cytotoxic effect of GPG gel is expected because of the toxicity of GPG for its effect on cell viability. At the highest concentration, blank gels displayed negligible cytotoxicity.

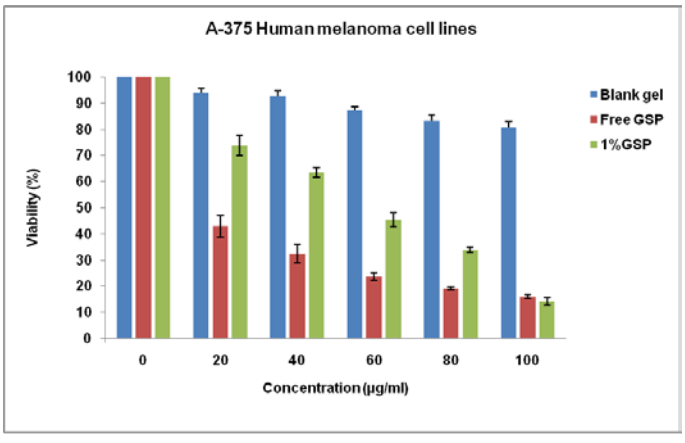

Fig. 6: *In vitro* cell viability data of GPG loaded gel and free GPG in A-375 human melanoma cell culture mean±SD(n=3)

Stability studies

As can be seen from the tests, gossypin flux from the vesicles may be due to a change in the surfactant and lipid phase at 40 °C (table 6). Be that as it may, there was no significant difference in vesicular size or entrapment efficiency (EE) formulations when they were removed after 90 d at 2-8 °C and 25±2 °C. To know the stability of optimised formulation after stability studies, a further similar cytotoxicity study was performed, which indicates that the formulation has no significant difference of cytotoxic effect against A-375 human cell lines was observed.

Table 6: Stability study of GPG-7

Storage time (days)	2–8 °C		25±2 °C		40 °C±2 °C	
	Vesicle size (nm)	EE (%)	Vesicle size (nm)	EE (%)	Vesicle size (nm)	EE (%)
0	189.3	95.5	189.3	95.5	189.3	95.5
30	189.2	95.2	189.0	95.1	186.2	94.0
60	189.0	94.8	189.2	95.0	186.5	92.1
90	188.9	94.6	189.0	95.2	186.0	91.3

Data expressed in mean±SD (n=3)

CONCLUSION

Surface morphology showed that the vesicles were nearly circular and clearly visible with sharp limits and large internal aqueous space. The test results showed that the EE percentage of prepared gossypin proniosomal gels is adequate (81.3 percent–95.5 percent) and they are Nano-sized (189.3–912.0 nm), and the gels dispersion gave the ideal continuing effect. Formulation GPG-7, containing the Span60 as a surfactant suggested a more prominent *in vitro* drug release, i.e. 75.5% of each surfactant. The formulation GPG-7 (mid-level Span 60) displayed 65.5 percent higher ex-vivo drug releases than other formulations with Span 20, Span 40 and Span 80. Gossypin loaded gels (GPG) showed 14.9±2.3 percent cell viability as compared to 16.1±1.1 percent non-Gossypin's most significant GPG dose of 100 µg/ml after 24h incubation for A-375 human melanoma cells. There was no significant improvement in the vesicular size or entrapment efficiency (percent EE) of formulations when stored at 2-8 °C and 25±2 °C for 90 d. This study demonstrated the reasonableness of proniosomal gel in achieving the ideal sustainability effect for gossypin's topical conveyance for melanoma therapy. The proniosomal gel improved both *in vitro* skin permeation and Gossypin deposition. It was shown to be skin-free and capable of holding Gossypin in the skin's deeper layers for an all-inclusive time. The findings also clearly defined that the Gossypin loaded proniosomal gel can be a good carrier option for transporting the drug through skin layers. More studies are necessary to ensure the long-term reliability of Gossypin loaded proniosomal gel and the absence of cytotoxicity on the various organs to use this carrier may be used to treat skin malignant development. Our findings can open the conceivable results for GPG topical delivery to treat skin-malignant tissues.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTERESTS

Declared none

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